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Differential Flotation Centrifugation Study of Hepatitis C Virus and Response to Interferon Therapy

A. Nagasaka,[1]* S. Hige,[1] T. Matsushima,[2] J. Yoshida,[1] Y. Sasaki,[1] I. Tsunematsu,[1] and M. Asaka[1]

[1]Third Department of Internal Medicine, Hokkaido University School of Medicine, Sapporo, Japan
[2]Department of Gastroenterology, Hakodate Municipal Hospital, Hakodate, Japan

Hepatitis C virus (HCV) appears to circulate in various forms such as native virion, immune complexes, and nucleocapsids during chronic infections. To determine the association of the physicochemical properties of HCV and its response to interferon therapy in patients with chronic hepatitis C, we examined pretreatment serum samples from 43 patients with HCV RNA who had received interferon therapy, using differential flotation centrifugation in a NaCl solution with a density of 1.063 g/ml. After centrifugation, the ratio of HCV RNA in the top and bottom fractions was determined by the polymerase chain reaction and expressed as T/B. Patients with a sustained response to IFN therapy were found to have higher T/B ratios than transient responders who relapsed after treatment ($P < 0.01$) and nonresponders ($P < 0.01$). With regards to HCV genotypes, patients with genotype 1b had higher T/B ratios in the sustained response group than in the nonsustained response groups ($P = 0.001$), but patients with genotype 2 had a similar distribution of T/B among the 3 response groups (not significant). These findings indicate that the physicochemical properties of HCV affect the effectiveness of interferon therapy, particularly in patients with genotype 1b. ***J. Med. Virol. 52:190–194, 1997.***

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INTRODUCTION

Although chronic hepatitis C may follow a mild and asymptomatic course, it can lead to cirrhosis and hepatocellular carcinoma in a substantial proportion of patients [Hopf et al., 1990; Saito et al., 1990]. At present, interferon (IFN) has been shown to be effective in normalizing transaminase and reducing the amount of viral replication in some patients [Shindo et al., 1991; Brillanti et al., 1991; Magrin et al., 1992]. Unfortunately, many patients receiving IFN therapy are resistant to treatment [Davis et al., 1989; Di Bisceglie et al., 1989; Tine et al., 1991]. Recent studies have identified viral subtype and serum viral levels as the most important pretreatment predictive factors [Yoshioka et al., 1992; Lau et al., 1993; Mita et al., 1994; Hagiwara et al., 1993; Tsubota et al., 1994]. However, these are not accurate enough to predict the effectiveness of IFN therapy.

The buoyant density of hepatitis C virus (HCV) has been estimated to be approximately 1.08 g/ml by sucrose density gradient equilibrium centrifugation. [Bradley et al., 1991; Miyamoto et al., 1992; Kanto et al., 1994; Hijikata et al., 1993]. Hijikata et al. [1993] found that there is a close relationship between in vivo infectivity and the buoyant density. They showed that HCV with high infectivity is related to low density and that low infectivity is related to high density. Furthermore, using differential flotation centrifugation and immunoprecipitation, they found that HCV in the bottom fraction was bound to anti-HCV antibodies as immune complexes, which have low infectivity.

Little is known about the relationship between the physical properties of HCV and clinical course. In the present study we determined the physical properties of HCV using differential flotation centrifugation and its relationship to the responsiveness to IFN therapy.

MATERIALS AND METHODS

Patients

Forty-three patients with chronic hepatitis C as confirmed by the presence of anti-HCV antibodies (EIA2, Abbott Laboratories, North Chicago, IL), HCV-RNA in the serum, and liver biopsies had been treated with IFN-α (6MU daily for 4 weeks, followed by 3 times

*Correspondence to: Atsushi Nagasaka, Third Department of Internal Medicine, Hokkaido University School of Medicine, Kita 15 Nishi 7, Sapporo 060, Japan.

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weekly for 5 months). The patients were followed up for at least 6 months after treatment. The response to IFN was divided into 3 groups as sustained response (SR), transient response (TR) and nonresponse (NR). SR indicated that normal alanine aminotransferase (ALT) levels were maintained for at least 6 months after treatment and that the HCV RNA was not found 6 months after treatment. TR indicated that ALT values were normal at the end of therapy but relapsed afterward. NR indicated that ALT values were abnormal continuously. HCV RNA was found at 6 months after treatment in both the TR and NR groups.

Differential Flotation Centrifugation

Differential flotation was carried out according to the method described by Hijikata et al. [1993]. This method was designed originally for fractionation of plasma low-density lipoprotein by Havel et al. [1995]. Fifty microliters of sera obtained at the pretreatment stage were mixed with 8 ml of NaCl solution with a density of 1.063 g/ml and centrifuged in a Hitachi 70P-73 rotor at 139,500 g for 22 h at 14°C. After centrifugation, 1 ml of the top and bottom fractions were collected.

Reverse Transcription (RT)/Polymerase Chain Reaction (PCR)

The HCV RNA was extracted from 100 μl of each fraction using a commercial kit (Sepa Gene-RV; Sanko Junyaku Co., Ltd., Japan). The HCV RNA pellets were then dissolved in 10 μl of distilled water and mixed with Molony murine leukemia virus reverse transcriptase (GIBCO BRL, Gaithersburg, MD), RNase inhibitor (Promega, Madison, WI), and random primer (Takara Biomedicals, Kyoto, Japan) to a final volume of 20 μl. The HCV RNA solution was incubated for one hour at 37°C to convert it to complementary DNA (cDNA). The HCV-cDNA was then amplified with the polymerase chain reaction (first-round PCR) using the following procedures: 2.5 μl of the cDNA was added to the reaction mixture (10 mmol/L Tris-HCl, pH 8.3, 2.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 200 μmol/L deoxynucleotide triphosphate), 5 pmol each of the sense- and antisense-strand primers, and 1 unit of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) to a final volume of 25 μl. In general, each reaction cycle was carried out at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. A total of 40 reaction cycles were performed. The sense primer used was 5′TCACTCCCCTGTGAGGAACT3′, and the anti-sense primer used was 5′TGCACGGTCTACGAGACCTC3′. These were designed on the basis of the sequence of the 5′ noncoding region of HCV-BK strain [Takamizawa et al., 1991]. PCR products were stained with ethidium bromide and observed under UV light.

When the HCV RNA in both of the fractions could not be detected, a second PCR was performed using a nested primer. The sense primer used was 5′TTCACGCAGAAAGCGTCTAG3′ and the anti-sense primer used was 5′GTTTATCCAAGAAAGGACCC3′. One microliter of the first PCR product was mixed with the same parameters and carried out under the same conditions as the first-round PCR.

In cases where the HCV RNA appeared in both fractions, the ratio of titers in the top and bottom fractions was determined as follows: cDNA in the top and bottom fractions was diluted serially in 10-fold increments and RT/PCR was then carried out. The ratio of titers in the top and bottom fractions was expressed as T/B.

Other methods. The HCV genotypes were determined by a two-stage PCR using mixed primers that were derived from the putative core region according to the method of Okamoto et al. [1992]. Nomenclature of genotype was subjected to a system described by Simmonds et al. [1993]. The HCV RNA was quantitated in the pretreatment serum by signal amplification employing branched DNA (bDNA) in a sandwich hybridization assay according to the manufacturer's instructions (Quantiplex Version 1.0; Chiron Corp., Emeryville, CA).

The statistical significance of differences was determined by one-factor ANOVA, the Mann-Whitney U test or the Kruskal-Wallis test when comparing group frequencies as appropriate, or by Fisher's exact probability test when comparing percentages. Correlation between HCV RNA levels and the T/B ratios was assessed using Spearman's test. To ensure that any association was not dependent at the computation level, the analysis was done twice with the bDNA negative group set at zero and 10^4 level as previously reported by Lau et al. [1993]. To determine the independent prognostic value of the selected factors, a multiple regression model was used. A P value of less than 0.05 was considered statistically significant.

RESULTS

The clinical background, genotypes, and HCV RNA levels among the 3 response groups are summarized in Table I. There were no significant differences among the 3 response groups with regards to age, sex, ALT levels found at the pretreatment stage, and liver histology. The 19 patients destined to have a sustained response to IFN had lower viremia levels before treatment began (median below cut-off, range up to 1.9 Meq/ml) than the 17 nonresponders (median 1.5, range cut-off to 24 Meq/ml; $P < 0.001$). There was no difference in the viremia levels between the nonresponders and the 7 transient responders (median 0.6, range cut-off to 13 Meq/ml).

There was a significant difference among the 3 response groups and their HCV genotypes as per the Kruskal-Wallis test ($P < 0.05$). Nine of the 11 patients (81.8%) who had genotype 2a showed a sustained response to IFN compared with 9 of the 29 patients (31%) who had genotype 1b ($P < 0.01$).

T/B Ratios, Immunological Data, Amount of Virus, and Response to IFN

T/B ratios could be determined in 40 cases at the first PCR. Only 3 cases required a second PCR. T/B ratios

TABLE I. Clinical Characteristics and HCV-Related Markers of the Patients Studied

	SR (n = 19)	TR (n = 7)	NR (n = 17)
Age[a]	47 ± 13	50 ± 12	46 ± 12
Sex (M/F)	11/8	5/2	12/5
ALT (IU/L)[a]	140 ± 129	102 ± 114	175 ± 120
Stage of fibrosis			
mild	2	1	2
moderate	15	5	6
severe	2	1	9
HCV RNA levels (Meq/ml)			
<0.5	15	3	4
0.5–1.0	2	1	1
>1.0	2	3	12
Genotype			
1b	9	5	15
2a	9	1	1
2b	1	1	1

SR, sustained response; TR, transient response; NR, nonresponse; ALT, alanine aminotransferase.
[a]Data expressed as mean ± SD.

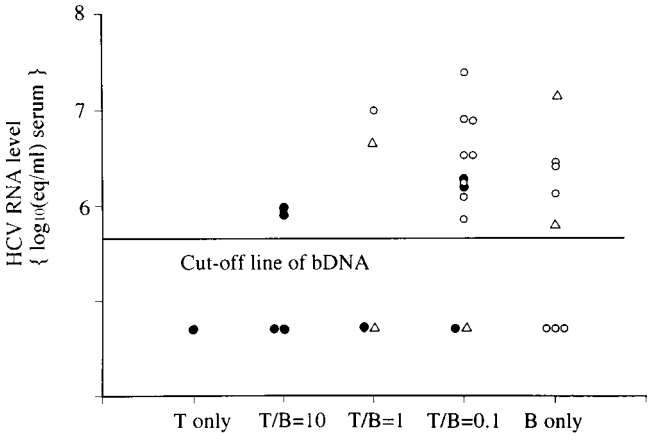

Fig. 1. The relationship in each IFN responder with genotype 1b between the HCV RNA levels and the T/B ratios. T/B, the ratio of HCV RNA in the top and bottom fractions; eq, equivalents. HCV RNA level is measured with branched DNA assay (bDNA) and the detection limit of this assay is 500,000 equivalents/ml. There was no significant correlation between the two (Spearman's rank correlation). The closed circles, the triangles, and the open circle are the sustained, the transient and the nonresponders, respectively.

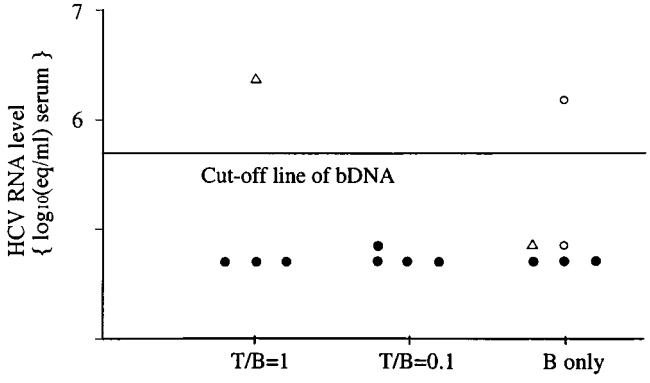

Fig. 2. The relationship in each IFN responder with genotype 2 between the viremic levels and the T/B ratios. Abbreviations are explained in the legend for Fig. 1. There was no significant correlation between the two (Spearman's rank correlation).

had no correlation to EIA2 titers and serum IgG levels (Table II). We also compared the T/B ratios with the amount of virus measured by bDNA assay. The relation of the T/B ratios to the amount of virus in each response are shown in Figures 1 and 2. Figure 1 shows it in patients with genotype 1b and Figure 2 in those with genotype 2. There were no significant relationships between the T/B ratios and the viremic levels in genotype 1b and 2 (Spearman's rank correlation). Tables III and IV also summarized the relationship between the T/B ratios and the 3 response groups. In Table III (genotype 1b), 6 of the 9 (66.7%) sustained responders showed T/B $\geq$ 1.0 and none showed B only, whereas 17 of the 20 (85%) unsustained responders showed T/B < 1.0. There were significant differences between the SR and the non-SR groups ($P = 0.001$). Furthermore, in the patients with genotype 1b and a negativity of bDNA (Fig. 1), the T/B ratios were also found to be significantly higher in the SR group than in the non-SR groups ($P < 0.05$). In the genotype 2 group (Table IV), contrary to genotype 1b, 7 of the 10 (70%) sustained responders showed T/B < 1.0 and were similar in their distributions of T/B to the unsustained responders. In Figure 2, all patients with SR had low viremic loads despite the T/B ratios.

To investigate further whether the T/B ratios were an independent predictive factor of IFN therapy in the patients with genotype 1b, we examined multivariate analysis consisting of seven factors (Table V). The only significant predictive factor was the T/B ratio ($P = 0.0122$).

TABLE II. Relationship Between T/B and Immunological Data*

T/B	EIA2	IgG (mg/dl)
T > B (n = 5)	5.581 ± 0.325	2261.5 ± 320.1
T = B (n = 8)	5.530 ± 0.505	2083.5 ± 871.8
T < B (n = 30)	5.691 ± 0.606	2003.2 ± 640.5

*T/B is the ratio of HCV RNA in the top and bottom fraction.
Data were expressed by mean ± SD.
There are no significant differences among T/B ratios (one-factor ANOVA).

DISCUSSION

We show that the response to IFN increased with the prominence of viruses in the lipid (top) fraction. The patients with genotype 1b in the SR group had a larger amount of virus in the top fraction than those with genotype 1b in the non-SR groups. On the other hand, the patients with genotype 2 had a larger amount of virus in the bottom fraction despite their response to IFN. This indicates that the patients with genotype 2 respond to IFN therapy in poor relation to the T/B ratios, but those with genotype 1b responded to it in close relationship to the T/B ratios.

The pretreatment viremia levels and HCV genotypes seem to be the most important predictive factor of response to IFN [Yoshioka et al., 1992; Lau et al., 1993;

TABLE III. Relationship Between T/B and Response to IFN in Patients With Genotype 1b*

	Response to IFN		
T/B	SR (n = 9)	TR (n = 5)	NR (n = 15)
T only	1	0	0
T/B = 10	4	0	0
T/B = 1	1	2	1
T/B = 0.1	3	1	8
B only	0	2	6

*T/B is the ratio of HCV RNA in the top and bottom fractions; IFN, interferon; SR, sustained response; TR, transient response; NR, nonresponse.
T/B ratios were significantly higher in the SR group than in the NR group ($P = 0.001$).

TABLE IV. Relationship Between T/B and Response to IFN in Patients With Genotype 2*

	Response to IFN	
T/B	SR (n = 10)	non-SR (n = 4)
T only	0	0
T/B = 10	0	0
T/B = 1	3	1
T/B = 0.1	4	0
B only	3	3

*T/B is the ratio of HCV RNA in the top and bottom fractions. IFN, interferon; SR, sustained response.

TABLE V. Multivariate Analysis of Variables Contributing to Responses to IFN in Genotype 1b

Variable	*P* value
T/B ratio[a]	0.0122
Stage of fibrosis (mild vs. moderate vs. severe)	0.3271
Total dose of interferon	0.3415
HCV RNA levels (Meq/ml) (<0.5 vs. 0.5–1.0 vs. ≥1.0)	0.4701
Sex (male vs. female)	0.5420
Age	0.6759
Alanine aminotransferase levels	0.9850

[a]Variable of T/B ratio: T only vs. 10 vs. 1 vs. 0.1 vs. B only.
All variables data were determined at pretreatment.

Mita et al., 1994; Hagiwara et al., 1993; Tsubota et al., 1994]. In particular, the bDNA method, which is easiest to perform, is inherently quantitative and is a good predictive marker for potential response to IFN therapy [Lau et al., 1993], confirming our present findings. However, 14 of the 20 patients whose bDNA was negative had a sustained response. Our result of a close relationship between the T/B ratios and the response to IFN in the patients with genotype 1b (Table V) provides evidence that the T/B ratios are a significant independent predictor of a sustained response to IFN not influenced by viremia levels.

The reason that the response to IFN in the patients with genotype 1b was influenced by the T/B ratios is not known. It seems that the top fraction contains native virion and the bottom fraction contains nucleocapsids and/or immune complexes. Therefore, a patient with a T/B < 1.0 seems to be under a significant humoral response. However, we could not find any correlation between the T/B ratios and EIA2 titers, serum IgG levels. Further studies on immunological factors such as anti-envelope antibodies will be necessary. HCV E1 and E2/NS1 encode the putative viral envelop proteins. Comparison of the available E2/NS1 sequences revealed the presence of a hypervariable region [Hijikata et al., 1991; Kato et al., 1992]. Specific antibody reactions were detected against peptides corresponding to the linear epitopes in HVR1, indicating that this HVR1 encodes antigenically distinct variants, which are subjected to immune selection [Weiner et al., 1992]. The observed hypervariability may result from sequential mutations leading to escape mutants [Kato et al., 1993]. Recently it has been reported that the heterogeneity of HVR1 quasispecies may be a factor for the potential predicting response to IFN therapy in patients with chronic hepatitis C [Okada et al., 1992; Kanazawa et al., 1994; Moribe et al., 1995]. A high level of humoral immune interference may introduce a high complexity of the HVR1 and increasing nucleocapsids and/or immune complexes in circulation which correspond to HCV in the bottom fraction and may interrupt the IFN reaction. Another possible explanation is that a patient who has low levels of cytotoxic T cell (CTL) activity will have a poor response to IFN. Löhr et al. [1994] demonstrated that the humoral immune response to HCV had a pathogenetic significance and was a predictive factor for response to IFN-α. B cell activation appears to suppress the CTL differentiation because of activated cytokines such as IL-10. To eliminate the virus by IFN, sufficient CTL response in the host may be required. On the other hand, the reason patients with genotype 2 had a good response despite the T/B ratios may be explained that genotype 2 HCV has a higher sensitivity to IFN than genotype 1 HCV.

Finally, this study indicates that the physicochemical properties of HCV are associated with the effectiveness of IFN therapy, particularly in patients with genotype 1b. On the other hand, patients with genotype 2 respond well to IFN despite the T/B ratios. The host immune reaction may affect the response to IFN in addition to viral factors such as viral loads and genotypes.

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